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14. ABSTRACT

The epidermal growth factor (EGF) family ligand amphiregulin (AR) has been associated with breast cancer. We demonstrate that EGF-independent SUM149 breast cancer cells are synthesizing and secreting AR. MCF10A human mammary epithelial cells made to over express AR (MCF10A AR) are also EGF-independent for growth. Treatment with the pan-erbB inhibitor CI1033 and the anti-EGFR antibody C225 demonstrated that ligand mediated activation of EGFR is required for SUM149 cell proliferation. AR neutralizing antibody reduced both SUM149 EGFR activity and cell proliferation confirming that an AR autocrine loop is required for mitogenesis. EGFR tyrosine phosphorylation was dramatically decreased in both SUM149 and MCF10A AR cells after inhibition of AR cleavage, indicating that an AR autocrine loop is strictly dependent on AR cleavage in culture. However, a juxtacrine assay where fixed SUM149 cells and MCF10A AR cells were overlain on top of EGF starved MCF10A cells showed that the AR membrane precursor can activate EGFR. SUM149 cells, MCF10A AR cells and MCF10A cells growing in exogenous AR were all considerably more invasive and motile than MCF10A cells grown in EGF. Moreover, AR upregulates genes involved in motility and invasion suggesting AR contributes to breast cancer progression.

15. SUBJECT TERMS
Amphiregulin, Epidermal Growth Factor Receptor, Autocrine Loop, Breast Cancer

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Annual Summary for Award W81XWH-06-1-0405
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Introduction:

The epidermal growth factor receptor (EGFR)[1], or erbB1, is a transmembrane protein possessing intrinsic tyrosine kinase activity. There are several EGF family ligands that can bind and activate the EGFR including epidermal growth factor (EGF) (1), amphiregulin (AR) (2), heparin binding epidermal growth factor (HB-EGF) (3), epiregulin (EPR) (4), betacellulin (BTC) (5), transforming growth factor-alpha (TGF-α) (6), and epigen (7). Ligand binding facilitates dimerization of the EGFR which activates downstream pathways known to be involved in cell growth, proliferation, differentiation, and migration (8).

Each EGF family ligand is expressed as a transmembrane precursor which is proteolytically cleaved and released into the external milieu. There is no obvious homology in the predicted cleavage sites of the EGFR ligands, but it has been shown that metalloprotease activity is required for their release (9-12). The identities of the proteases involved in ligand cleavage are still obscure but there is considerable evidence to suggest that TNF-α converting enzyme (TACE/ADAM 17) is involved specifically in AR, HB-EGF, and TGF-α cleavage (9, 10). Although it is well known that soluble growth factors are biologically active, there is substantial debate in the current literature about the activity of EGF family precursor proteins. Certain EGF family members such as HB-EGF, AR, TGF-α, and Betacellulin have been suggested to activate EGFR via juxtacrine interactions while membrane bound EGF has been shown to lack activity (13-19).

AR was originally purified from the conditioned media of MCF-7 breast cancer epithelial cells treated with the tumor promoter phorbol 12-myristate-13-acetate (PMA) (20). It is synthesized as a 252 amino acid heparin binding glycoprotein with an EGF-like domain and a basic NH2 terminus which contains glycosylation sites and putative nuclear localization signals (21). Due to differential processing and glycosylation, many different sizes of membrane anchored AR (16-50 kDa) and secreted AR (60-9 kDa) have been found (2, 11, 20-24). *In vivo*, AR mRNA is expressed in many normal tissues including placenta, testis, pancreas, spleen, kidney, lung, breast, ovary, and colon (21). AR activation of EGFR appears to play a particularly relevant role in the developing breast since AR is the critical EGFR ligand required for ductal morphogenesis in the mouse mammary gland and it has been shown to act as an autocrine growth factor for some normal human mammary epithelial cells (25, 26) .

There is direct evidence that an EGFR/AR autocrine loop exists in pancreatic cancer, colon cancer, and hepatocellular carcinoma (27-29). AR expression was also found to be strongly correlated with inflammatory breast cancer (IBC) and a putative AR/EGFR autocrine loop is suggested to contribute to breast cancer progression (30). Indeed AR may play a specific role in cancer progression as it has been shown that AR activation of EGFR contributes to the synthesis, secretion and activation of some proteins involved in invasion and metastasis such as uPa, MMP-9 and EMMPRIN (31-33).

We have developed the cell line SUM149 in our laboratory from an aggressive inflammatory breast cancer and we used this cell line as a model to study the mechanism of AR action and its potential role as an autocrine growth factor in breast cancer (34). SUM149 cells are estrogen receptor negative and over express EGFR but do not express any other active erbB family members. For preliminary data shown in our proposal, we demonstrated that SUM149 cells are synthesizing and secreting AR which is dependent on EGFR activity. In addition, these cells depend on AR for their proliferation. Therefore, SUM149 cells have an amphiregulin/EGFR autocrine loop which is suggested to be involved in tumor progression.

MCF10A cells are immortalized, non-transformed human mammary epithelial cells with an obligatory requirement for EGF for their growth and proliferation (35). We have shown that over expression of AR in MCF10A cells (MCF10A AR) renders them EGF-independent for cell proliferation. Although, the ability of membrane bound AR to signal through the EGFR is still not clear due to contradicting evidence in the literature, our data show that both SUM149 cells and MCF10A AR cells have large amounts of AR on the membrane which suggests that AR precursor is activating EGFR and contributing to the maintenance of an autocrine loop. Since AR expression is strongly correlated with aggressive breast cancer, this suggests that there is a connection between AR signaling and the aggressive breast cancer phenotype. Therefore, we hypothesize that AR signaling through the EGFR contributes to the expression of genes involved in the progression of inflammatory breast cancer.

Body:

Task 1: To directly test the hypothesis that membrane bound amphiregulin can activate EGFR and is required for the maintenance of an amphiregulin autocrine loop:

a. Perform juxtacrine growth factor activity assays using SUM149 and MCF10A AR cells to determine how membrane bound amphiregulin affects EGFR phosphorylation and proliferation of MCF10A cells grown without EGF (Months 10-15).

Pro-AR is a membrane anchored precursor that is either cleaved to produce a soluble form that can activate EGFR or may possibly signal via juxtacrine interactions with EGFR (15, 36). It is still not clear whether the AR precursor is biologically active but there is some evidence to suggest that AR can signal in a juxtacrine fashion (15). SUM149 breast cancer cells express significant amounts of membrane bound AR which may contribute to their ability to maintain an AR autocrine loop if the precursor form is biologically active. Therefore, we performed studies to investigate the potential mechanism of EGFR activation by membrane bound AR.

To determine whether uncleaved AR is able to activate EGFR in a juxtacrine fashion, we performed a juxtacrine assay modified from the assay originally performed by Takemura et al. which examined the signaling ability of membrane precursor HB-EGF (37). MCF10A cells, which express relatively high levels of EGF receptors, were grown without EGF for 24 hours to decrease EGFR tyrosine phosphorylation. SUM149 and MCF10A AR cells were detached from confluent plates using EDTA and then acid washed three times to remove cleaved cell-associated AR, leaving only membrane precursor AR on the cell surface. Following the acid washes, SUM149 and MCF10A AR cells were formalin fixed and overlain on top of the EGF starved MCF10A cells for 5 minutes. Subsequent immunoprecipitation of EGFR followed by SDS-PAGE separation and immunoblotting with a phosphotyrosine antibody showed dramatically increased tyrosine phosphorylation of EGFR following exposure to fixed SUM149 cells and MCF10A AR cells but not with MCF10A cells that had been grown in their normal EGF-containing media (Figure 6). When AR neutralizing antibody was added to the overlain SUM149 cells and MCF10A AR cells, EGFR tyrosine phosphorylation was significantly reduced, suggesting that membrane precursor AR in both SUM149 cells and MCF10A AR cells activated EGFR on MCF10A cells via juxtacrine interactions (Figure 1).

Figure 1

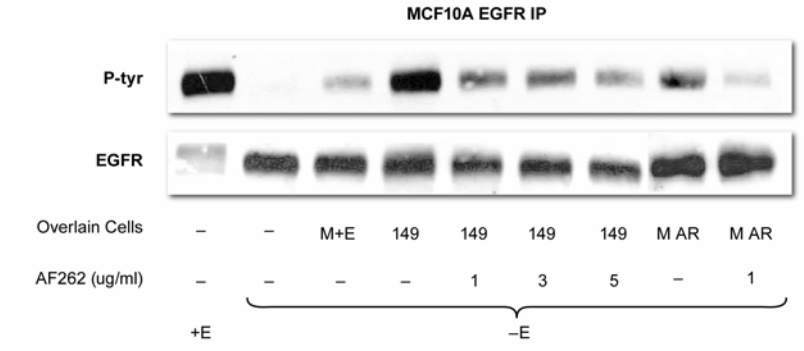

Figure 1: SUM149, MCF10A, or MCF10A AR cells were removed from confluent plates, acid washed three times, fixed with formalin and resuspended in SFIH media. The cells were incubated with or without AR neutralizing antibody AF262 for 5 minutes at concentrations of 1μg/ml, 3μg/ml, or 5μg/ml as indicated. Fixed cells were then overlain on top of EGF starved MCF10A cells for 5 minutes. Western blot shows EGFR tyrosine phosphorylation after an MCF10A EGFR immunoprecipitation.

b. Perform assays using Batimastat as an inhibitor of amphiregulin secretion in SUM149 cells and MCF10A cells in order to determine the requirement of membrane bound amphiregulin for an autocrine loop (Months 1-4).

In an effort to determine the requirement of AR cleavage for EGFR activity in SUM149 cells, SUM149 cells were treated with the broad spectrum metalloprotease inhibitor GM6001. Although we originally proposed to use the broad spectrum metalloprotease inhibitor Batimastat, we changed to GM6001 as Batimastat is no longer being sold and GM6001 has been shown to block AR cleavage in keratinocytes (38).

To confirm that GM6001 inhibits AR secretion in SUM149 cells, an ELISA was performed. SUM149 cells were subjected to one, two or three acid washes to remove cleaved AR attached to the heparan sulfate proteoglycan surface of the cell which could be released into the media and increase the total background AR concentration. These acid washes were then followed with DMSO control treatment or 20μM GM6001 for 5min, 15 min or 30 min. As observed with the DMSO controls, each consecutive acid wash removed more residual cleaved AR from the media. After 3 acid washes and GM6001 treatment, secreted AR in SUM149 conditioned media dropped to a concentration below 5pg/ml per 100,000 cells (Figure 2A). As a result, these data show that AR secretion is essentially completely blocked by GM6001 in SUM149 cells.

Figure 2

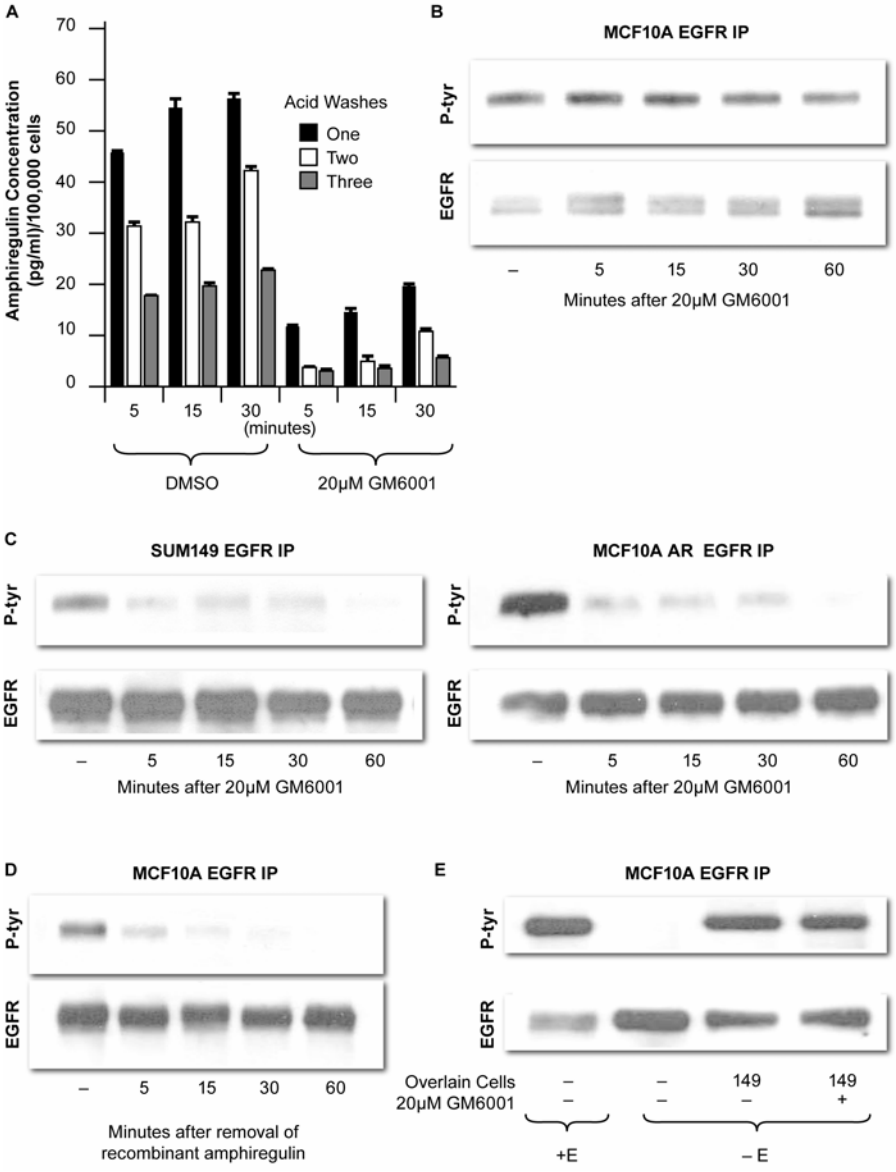

Figure 2: *A*) SUM149 cells were cultured in 6-well plates with fresh media. Cells were acid washed once, twice or three times and treated with either DMSO or 20μM GM6001 for 5, 15, 30 or 60 minutes. Conditioned media was measured for AR by an ELISA. Cells were counted for normalization using a coulter counter. Error bars represent the standard error of three replicate experiments *B*) Western blot after EGFR immunoprecipitation showing EGFR tyrosine phosphorylation in MCF10A cells that were acid washed three times and treated with 20μm GM6001 for 5, 15, 30 or 60 minutes. *C*) Western blot after EGFR immunoprecipitation showing EGFR tyrosine phosphorylation in SUM149 and MCF10A AR cells that were acid washed three times and treated with 20μm GM6001 for 5, 15, 30 or 60 minutes. *D*) Western blot after EGFR immunoprecipitation showing EGFR tyrosine phosphorylation in MCF10A cells that were grown in SFIHA media for one passage. AR was washed away and the cells were

cultured in SFIH media for 5, 15, 30 or 60 minutes. *E*) Juxtacrine assay after SUM149 cells were treated with 20μM GM6001 for 15 minutes.

In an effort to determine how blocking AR secretion affects EGFR activity, we acid washed MCF10A, MCF10A AR, and SUM149 cells three times to remove cleaved AR from the cell surface, and then exposed cells to 20μM GM6001 for varying times. GM6001 had no effect on exogenous EGF-induced tyrosine phosphorylation of EGFR, as EGFR phosphorylation was not reduced by GM6001 treatment of MCF10A cells cultured in the presence of EGF (Figure 2B). However, inhibition of AR secretion with GM6001 in SUM149 and MCF10A AR cells resulted in a significant decrease in EGFR tyrosine phosphorylation with very little residual phosphorylation remaining at 60 minutes (Figure 2C).

Given that AR cleavage was effectively blocked by GM6001 after only 5 minutes, it was possible that the residual EGFR activity we observed from 15 minutes to 60 minutes after GM6001 treatment in SUM149 and MCF10A AR cells was induced by the non–cleaved AR precursor on the cell surface. Alternatively, non-cleaved AR may have no ability to activate EGFR in a truly autocrine fashion, in which case the residual EGFR tyrosine phosphorylation observed would represent activated receptor that had not been completely degraded or dephosphorylated. To distinguish between these possibilities, MCF10A cells were cultured in AR-supplemented serum-free medium. We then examined how quickly EGFR activity decreases after removal of exogenous AR from the media. MCF10A cells were grown in 10ng/ml AR for one passage and AR was washed out for 5, 15, 30 or 60 minutes followed by EGFR immunoprecipitation and western analysis (Figure 2D). We found that EGFR tyrosine phosphorylation, after removal of soluble AR, decreased in a similar fashion to EGFR tyrosine phosphorylation in SUM149 and MCF10A AR cells after treatment with GM6001. Thus, the residual EGFR tyrosine phosphorylation we observed after GM6001 treatment appears to be activated receptor that had not been completely degraded or dephosphorylated. Altogether, our data demonstrate that while membrane precursor AR can activate EGFR via juxtacrine interactions, cleavage of AR plays a critical role in autocrine activation of EGFR.

The requirement for cleavage for autocrine activation of EGFR seemed incongruous with the observation that membrane precursor AR can activate EGFR in our juxtacrine assay. Together, these observations suggest that AR cleavage is required for autocrine activation of the EGFR, but not for juxtacrine activation of EGFR. To examine this further, and confirm that non-cleaved, membrane associated AR is able to activate EGFR, we repeated the juxtacrine experiment after blocking AR secretion with three acid washes followed by GM6001 for 15 minutes in the SUM149 cells. The data in figure 2E shows that inhibition of AR secretion using the metalloprotease inhibitor GM6001 did not effect activation of MCF10A EGFR by overlain SUM149 cells. Thus, we conclude that the increased EGFR tyrosine phosphorylation observed in MCF10A cells overlain with SUM149 or MCF10A AR cells is due to AR membrane precursor that interacts with, and activates the EGFR.

Task 3: To determine whether amphiregulin signaling through EGFR contributes to the inflammatory phenotype of Inflammatory Breast Cancer:

a. Analysis of motility and invasiveness of MCF10A AR cells (Months 15-20).

One of the hallmark characteristics of cancer cells is enhanced motility. To determine how an AR autocrine loop might contribute to the aggressive breast cancer phenotype, we investigated the effect of EGFR activation by AR on cell motility. We utilized a fluorescence cell motility assay in which the cells phagocytose blue fluorescent beads in their path, leaving black tracks behind as they move (Cellomics). We found that SUM149 cells, which over express AR, displayed an increase in motility compared with MCF10A cells (Figure 3A). In addition, both exogenous AR (MCF10A + AR) and over expression of AR (MCF10A AR) significantly stimulated directional motility compared with MCF10A cells grown in EGF containing media suggesting that an AR autocrine loop specifically contributes to the activation of genes involved in cell motility.

Figure 3

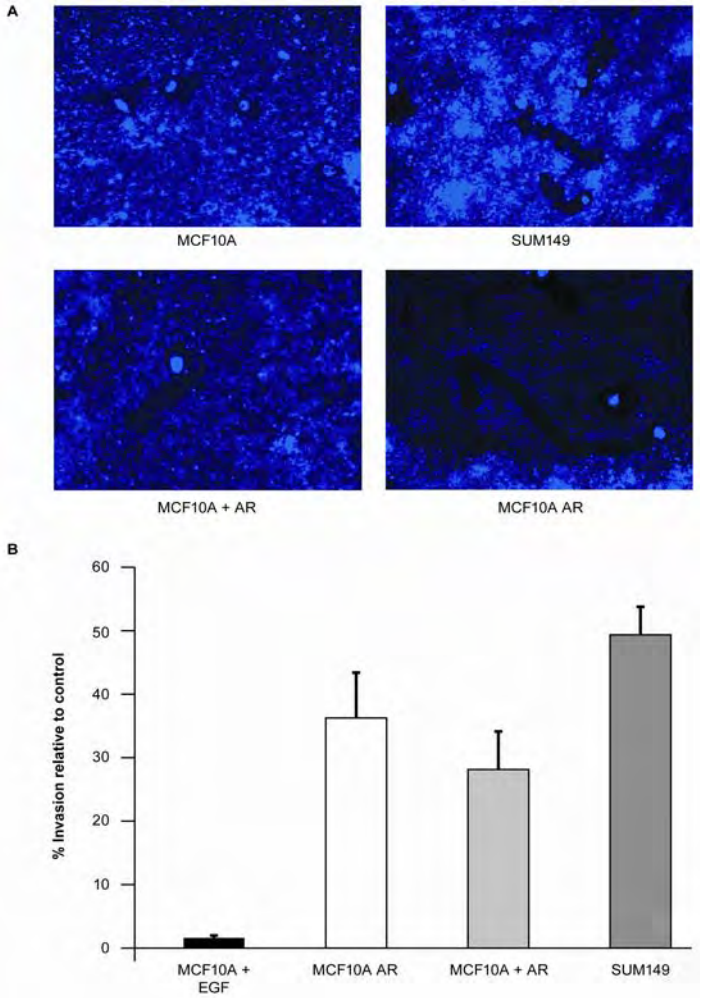

Figure 3: *A*) Cell motility assay images showing black tracks made by MCF10A cells grown in EGF (MCF10A), MCF10A cells grown in AR (MCF10A + AR), SUM149 cells, and MCF10A AR cells. Pictures were taken at 20x magnification after 24 hours. *B*) % invasion of MCF10A cells grown in EGF (MCF10A), MCF10A cells grown in AR (MCF10A + AR), SUM149 cells, and MCF10A AR cells was calculated by dividing the average number of cells on the membrane of a matrigel chamber after 24 hours by the

average number of cells on the membrane of a control chamber after 24 hours. Error bars represent the standard error of three replicate experiments.

Penetration of the extracellular matrix and the basement membrane is a key step in tumor dissemination and invasion. Therefore, we investigated the effect of AR on cell invasion in a modified Boyden chamber assay in which cells migrate through matrigel and a polycarbonate filter (Becton Dickinson). We evaluated the invasive capacity of SUM149 cells, MCF10A AR cells and MCF10A cells grown with either exogenous EGF or exogenous AR by determining the number of cells that penetrated the Matrigel. We found that MCF10A cells grown with EGF lack the ability to invade whereas MCF10A cells grown with AR were approximately 25% invasive. In addition, MCF10A AR cells were close to 35% invasive suggesting that AR activation of EGFR, but not EGF activation of EGFR, increases cell invasion. SUM149 cells were approximately 50% invasive in this assay (Figure 3B). Therefore, these data strongly indicate that an AR autocrine loop specifically contributes to the aggressive growth potential of SUM149 breast cancer cells.

b. Expression profiling of MCF10A cells stimulated by either EGF or amphiregulin using microarray technology followed by QPCR and western analysis to confirm microarray results (Months 4-14).

In an effort to determine which genes might be contributing to the increased cell invasion and motility of MCF10A cells growing with AR stimulated EGFR, we performed an expression array analysis using an Affymetrix human array platform comparing MCF10A cells versus SUM149 cells or MCF10A AR cells. Analysis of the results indicated that 97 genes were increased in their expression in both SUM149 and MCF10A AR cells relative to MCF10A cells (data not shown). Further analysis of the biological pathways using the data mining database Pathway-Express demonstrated that most of these genes fell into the major biological processes of toll-like receptor signaling pathway, apoptosis, MAPK signaling pathway, focal adhesion, and cytokine-cytokine receptor interaction (39). Annotation analysis using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) showed that 14 genes of the 97 upregulated genes were involved in processes such as cell adhesion, proteolysis, and chemotaxis which are related to cell motility and invasion (Table 1) (40).

TABLE 1

Differentially Regulated Genes involved in Cell Motility and Invasion

Gene Accession Number	Gene Name	Fold Change	Annotation
NM_005823	MSLN (Mesothelin)	55.72	Cell Adhesion
BC012501	NID (Nidogen)	19.70	Cell Matrix Adhesion; extracellular matrix structural constituent; calcium ion binding; cell adhesion molecule activity
M15329	IL1A (Interleukin 1 alpha)	18.38	Negative regulation of cell proliferation; anti-apoptosis; chemotaxis; immune response; regulation of cell cycle; cell-cell signaling; inflammatory response; interleukin-1 receptor binding; signal transducer activity
AI922855	CPE (carboxypeptidase 3)	11.31	Metabolism; protein modification; proteolysis and peptidolysis; neuropeptide signaling pathway; metallopeptidase activity
NM_006307	SRPX (sushi-repeat-containing protein X linked)	5.66	Cell adhesion
AW002864	AAD2 (Adducin 2 beta)	5.66	Actin binding; calmodulin binding
U07820	CNTN1 (Contactin 1)	5.28	Cell adhesion
NM_000576	IL1B (Interleukin 1 Beta)	4.92	Negative regulation of cell proliferation; apoptosis; immune response; signal transduction; regulation of cell cycle; cell-cell signaling; inflammatory response; antimicrobial humoral response; interleukin-1 receptor binding; signal transducer activity
NM_024022	TMPRSS3 (transmembrane protease, serine 3)	4.92	Hearing; proteolysis and peptidolysis; trypsin activity; scavenger receptor activity; chymotrypsin activity; hydrolase activity
BC002690	KRT14 (Keratin 14)	4.29	Epidermal differentiation; cell shape and cell size control; structural constituent of cytoskeleton
AI653981	L1CAM (Cell Adhesion Molecule)	2.64	Neurogenesis; cell adhesion molecule activity
U61276	JAG1 (Jagged 1)	2.14	Cell communication; Notch binding; calcium ion binding; growth factor activity; structural molecule activity
NM_006255	PRKCH (Protein Kinase C, eta)	2.14	Protein amino acid phosphorylation; signal transduction
NM_002423	MMP7 (Matrix Metalloprotease 7)	2.00	Collagen catabolism; zinc ion binding; calcium ion binding; matrilysin activity; hydrolase activity

Our data suggest that these genes may play particularly important roles in the breast cancer phenotype when cancer cells are dependent on a functional AR/EGFR autocrine loop. The IL-1 pathway has been implicated in breast cancer progression and therefore we will be focusing on the IL-1A and IL-1B genes from this list for further studies (41, 42). Validation of IL-1A and IL-1B genes from this affymetrix array analysis via QPCR and western analysis is ongoing.

Key Research Accomplishments:

1. AR precursor protein on the surface of SUM149 breast cancer cells can activate EGFR in a juxtacrine manner but cleavage is required for activation of EGFR in monolayer.

2. Over expression of AR or growth in exogenous AR increases MCF10A motility and invasion but growth in EGF does not.

3. AR increases the expression of several genes involved in cell motility and invasion compared with EGF and therefore might contribute to breast cancer progression.

Reportable Outcomes:

Manuscripts:

Willmarth NE and Ethier SP. Autocrine and juxtacrine effects of amphiregulin on the proliferative, invasive, and migratory properties of normal and neoplastic human mammary epithelial cells. Journal of Biological Chemistry. 2006 Dec 8; 281(49): 37728-37737.

Abstracts:

Willmarth NE and Ethier SP. Involvement of membrane bound amphiregulin in maintenance of an autocrine loop in inflammatory breast cancer cells. 2006 American Association for Cancer Research Annual Meeting in Washington, D.C. April 1-5, 2006. (Poster Presentation)

Conclusions:

We have made significant progress in understanding the effect of a functional amphiregulin (AR) autocrine loop in breast cancer progression. We have shown that an AR autocrine loop is required for SUM149 breast cancer proliferation and that AR increases both motility and invasion of human mammary epithelial cells. AR precursor can activate EGFR in a juxtacrine fashion and therefore might contribute to the growth of breast cancer cells *in vivo*. Furthermore, AR increases the expression of several genes involved in cell motility and invasion compared with EGF which suggests that an AR autocrine loop can contribute to breast cancer progression.

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Appendix

Autocrine and juxtacrine effects of amphiregulin on the proliferative, invasive, and migratory properties of normal and neoplastic human mammary epithelial cells.

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Autocrine and Juxtacrine Effects of Amphiregulin on the Proliferative, Invasive, and Migratory Properties of Normal and Neoplastic Human Mammary Epithelial Cells*

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Amphiregulin (AR) autocrine loops have been associated with several types of cancer. We demonstrate that SUM149 breast cancer cells have a self-sustaining AR autocrine loop. SUM149 cells are epidermal growth factor (EGF)-independent for growth, and they overexpress AR mRNA, AR membrane precursor protein, and secreted AR relative to the EGF-dependent human mammary epithelial cell line MCF10A. MCF10A cells made to overexpress AR (MCF10A AR) are also EGF-independent for growth. Treatment with the pan-ErbB inhibitor CI1033 and the anti-EGF receptor (EGFR) antibody C225 demonstrated that ligand-mediated activation of EGFR is required for SUM149 cell proliferation. AR-neutralizing antibody significantly reduced both SUM149 EGFR activity and cell proliferation, confirming that an AR autocrine loop is required for mitogenesis in SUM149 cells. EGFR tyrosine phosphorylation was dramatically decreased in both SUM149 and MCF10A AR cells after inhibition of AR cleavage with the broad spectrum metalloprotease inhibitor GM6001, indicating that an AR autocrine loop is strictly dependent on AR cleavage in culture. However, a juxtacrine assay where fixed SUM149 cells and MCF10A AR cells were overlaid on top of EGF-deprived MCF10A cells showed that the AR membrane precursor can activate EGFR. SUM149 cells, MCF10A AR cells, and MCF10A cells growing in exogenous AR were all considerably more invasive and motile than MCF10A cells grown in EGF. Moreover, AR up-regulates a number of genes involved in cell motility and invasion in MCF10A cells, suggesting that an AR autocrine loop contributes to the aggressive breast cancer phenotype.

The epidermal growth factor receptor (EGFR),[2] or ErbB1, is a transmembrane protein possessing intrinsic tyrosine kinase activity. There are several EGF family ligands that can bind and activate the EGFR including epidermal growth factor (EGF) (1), amphiregulin (AR) (2), heparin-binding epidermal growth factor (HB-EGF) (3), epiregulin (4), betacellulin (5), transforming growth factor-α (TGF-α) (6), and epigen (7). Ligand binding facilitates dimerization of the EGFR, which activates downstream pathways known to be involved in cell growth, proliferation, differentiation, and migration (8).

Each EGF family ligand is expressed as a transmembrane precursor, which is proteolytically cleaved and released into the external milieu. There is no obvious homology in the predicted cleavage sites of the EGFR ligands, but it has been shown that metalloprotease activity is required for their release (9–12). The identities of the proteases involved in ligand cleavage are still obscure, but there is considerable evidence to suggest that tumor necrosis factor-α-converting enzyme/ADAM 17 is involved specifically in AR, HB-EGF, and TGF-α cleavage (9, 10). Although it is well known that soluble growth factors are biologically active, there is substantial debate in the current literature about the activity of EGF family precursor proteins. Certain EGF family members, such as HB-EGF, AR, TGF-α, and betacellulin, have been suggested to activate EGFR via juxtacrine interactions, whereas membrane-bound EGF has been shown to lack activity (13–19).

AR was originally purified from the conditioned media of MCF-7 breast cancer epithelial cells treated with the tumor promoter phorbol 12-myristate-13-acetate (20). It is synthesized as a 252-amino acid heparin-binding glycoprotein with an EGF-like domain and a basic NH_2 terminus, which contains glycosylation sites and putative nuclear localization signals (21). Due to differential processing and glycosylation, many different sizes of membrane-anchored AR (16–50 kDa) and secreted AR (9–60 kDa) have been found (2, 11, 20–24). *In vivo*, AR mRNA is expressed in many normal tissues, including placenta, testis, pancreas, spleen, kidney, lung, breast, ovary, and colon (21). AR activation of EGFR appears to play a particularly relevant role in the developing breast, since AR is the critical EGFR ligand required for ductal morphogenesis in the mouse mammary gland, and it has been shown to act as an autocrine growth factor for some normal human mammary epithelial cells (25, 26).

There is direct evidence that an EGFR/AR autocrine loop exists in pancreatic cancer, colon cancer, and hepatocellular carcinoma (27–29). AR expression was also found to be

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[2] The abbreviations used are: EGFR, epidermal growth factor receptor; AR, amphiregulin; EGF, epidermal growth factor; HB-EGF, heparin binding epidermal growth factor; TGF-α, transforming growth factor α; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; RT, reverse transcription.

strongly correlated with inflammatory breast cancer, and a putative AR/EGFR autocrine loop is suggested to contribute to breast cancer progression (30). Indeed, AR may play a specific role in cancer progression, since it has been shown that AR activation of EGFR contributes to the synthesis, secretion, and activation of some proteins involved in invasion and metastasis, such as urokinase-type plasminogen activator, matrix metalloproteinase 9, and extracellular matrix metalloproteinase inducer (31–33).

We have developed the cell line SUM149 in our laboratory from an aggressive inflammatory breast cancer, and we used this cell line as a model to study the mechanism of AR action and its potential role as an autocrine growth factor in breast cancer (34). SUM149 cells are estrogen receptor-negative and overexpress EGFR but do not express any other active ErbB family members. In this report, we demonstrate that AR functions as an autocrine growth factor for a breast cancer cell line and show that this self-sustaining autocrine loop is dependent on EGFR activity.

MCF10A cells are immortalized, nontransformed human mammary epithelial cells with an obligatory requirement for EGF for their growth and proliferation (35). We show that overexpression of AR in MCF10A cells renders them EGF-independent for cell proliferation and increases both their cell motility and invasion capabilities. Similarly, SUM149 cells show increased cell motility and invasion relative to MCF10A cells. In addition, we provide evidence that membrane-anchored AR precursor can activate EGFR in a juxtacrine fashion, and therefore membrane-anchored AR may play an important role in the maintenance of an AR autocrine loop in breast cancer.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—The antibodies used were mouse monoclonal anti-EGFR antibody Ab-5 (Oncogene) to immunoprecipitate EGFR, mouse monoclonal anti-EGFR antibody clone 31G7 (Zymed Laboratories, Inc.) to detect EGFR, anti-EGFR human (mouse) antibody clone 225 (C225) (Calbiochem) to block ligand binding to EGFR, goat polyclonal anti-AR antibody AF262 (R&D Systems) to detect AR and neutralize AR, mouse monoclonal antibody PY-20 (Calbiochem) to detect EGFR tyrosine phosphorylation, and mouse monoclonal anti-AR antibody MAB262 (R&D Systems) also used to neutralize AR. CI1033 was obtained from Pfizer, and GM6001 was obtained from Calbiochem. Recombinant AR was obtained from R&D Systems.

Cell Culture—AR-overexpressing MCF10A cells (MCF10A AR) were made as previously described (36). SUM149 cells were maintained in Ham's F-12 medium with 5% fetal bovine serum, 5 μg/ml insulin, 2 μg/ml hydrocortisone, 5 μg/ml gentamicin, and 2.5 μg/ml fungizone. The serum-free base medium for MCF10A and MCF10A AR cells was SFIH (Ham's F-12 with 1 μg/ml hydrocortisone, 1 mg/ml bovine serum albumin, 10 mM Hepes, 5 mM ethanolamine, 5 μg/ml transferrin, 10 nM triiodothyronine, 50 nM sodium selenate, 5 μg/ml gentamicin, 2.5 μg/ml fungizone, and 5 μg/ml insulin), and MCF10A cells required 10 ng/ml EGF (SFIHE). MCF10A + AR cells were MCF10A cells grown in SFIH medium with 20 ng/ml exogenous AR (SFIHA), which was found previously to be the biologically equivalent concentration to EGF for these cells (36). All cells were maintained in a humidified incubator at 37 °C and 10% CO_2.

Quantitative RT-PCR—Total RNA was isolated from subconfluent cell culture plates using an RNeasy kit (Qiagen), and cDNA was reverse-transcribed from 4 μg of total RNA using the Superscript II kit (Invitrogen). Primers used were designed and synthesized by the Applied Biosystems Assays-by-Design service. AR primer sequences were 5′-GTTACTGCTTCCAGGTGCTCTA for the forward primer and 5′-GTTACTGCTTCCAGGTGCTCTA for the reverse primer. The probe sequence was 5′-ACGGAGAATGCAAATATA. A glyceraldehyde-3-phosphate dehydrogenase primer set was used as control. Real time quantitative RT-PCR was performed in 96-well plates using Taqman Universal PCR Master Mix (Applied Biosystems) at the University of Michigan Comprehensive Cancer Center Affymetrix and cDNA Microarray Core Facility. The reactions were done in replicates of 6. Calculation of the $\Delta\Delta CT$ values was performed as previously described (37). Briefly, for each cell line, the average number of cycles for glyceraldehyde-3-phosphate dehydrogenase control primer reactions to reach threshold fluorescence was calculated and subtracted from the average number of cycles for AR primer reactions to reach threshold fluorescence. These values for SUM149 and MCF10A AR cells were then subtracted from MCF10A values. These differences were then raised to the -2 power. Data are represented as -fold change relative to MCF10A control.

Immunoprecipitation and Immunoblotting—The antibody anti-EGFR Ab-5 (Oncogene Research Products) was used for immunoprecipitation. Immunoprecipitation and Western blotting were performed as previously described (38). Protein from whole cell lysates was loaded onto 15% SDS-polyacrylamide gels for AR detection, and EGFR immunoprecipitates were loaded onto 7.5% SDS-polyacrylamide gels. After transferring proteins to polyvinylidene difluoride membranes, blots were probed with anti-EGFR antibody 31G7 (Zymed Laboratories), anti-phosphotyrosine antibody PY-20 (Calbiochem), or anti-AR antibody AF262 (R & D Systems) and visualized by enzymatic chemiluminescence (Pierce).

Enzyme-linked Immunosorbent Assay (ELISA)—Conditioned medium was obtained from cells grown in 6-well plates. An AR DuoSet ELISA from R & D Systems was used to measure AR medium concentration. High binding ELISA plates were coated with 3 μg/ml MAB262 monoclonal AR antibody in sterile phosphate-buffered saline (PBS) overnight at room temperature. Absorbance was measured on a VERSAmax microplate reader (Molecular Devices Corp.). Cells were lysed, and nuclei were counted with a Z1 Coulter Counter (Beckman Coulter) for normalization. Samples were done in triplicate.

Cell Proliferation Assays—Cells were seeded on day 0 in 6-well plates at $\sim 1.0\times10^4$ cells/well. Either 1 μg/ml AF262, 1 μg/ml MAB262, 1 μg/ml C225, or 1 μM CI1033 was added daily. After 5 or 7 days of treatment, plates were washed with PBS three times and agitated on a rocker table with 0.5 ml of a Hepes/$MgCl_2$ buffer (0.01 M Hepes and 0.015 M $MgCl_2$) for 5 min. Cells were then lysed for 10 min using a Bretol (ethyl hexa-

decyldimethylammonium) solution, and the nuclei were counted using a Z1 Coulter Counter (Beckman Coulter). Day 1 cells were counted for seeding counts. All experiments were done in triplicate.

Juxtacrine Assays—MCF10A cells, SUM149 cells, and MCF10A AR cells were removed from confluent plates using 10 mM EDTA, washed three times with an acid wash (pH 4.0 Hanks' balanced salt solution), and fixed in formalin for 10 min. After fixation, the cells were washed three times in SFIH medium. The fixed cells were then resuspended in SFIH medium with either PBS or 1 μg/ml AF262 for 5 min and then overlaid on top of MCF10A cells that had been grown in EGF-free medium for 24 h. After 5 min, the plates were washed three times with PBS, followed by EGFR immunoprecipitation and Western analysis.

Cell Motility Assay—The Cell Motility Bioapplication and Hitkit from Cellomics was used for these experiments. According to the manufacturer's instructions, collagen-I-coated 96-well plates were coated with prewashed blue fluorescent beads. ~500 cells/well of MCF10A, SUM149, MCF10A AR, and MCF10A cells growing in AR were added to the wells of the plate. The plate was incubated for 24 h at 37 °C in 10% CO_2. UV images were taken at ×20 using a Nikon inverted microscope.

Cell Invasion Assay—Matrigel invasion chambers (BD Biosciences) were rehydrated with Dulbecco's modified Eagle's medium for 1 h in a 37 °C incubator. ~2.5×10^5 of MCF10A cells in SFIHE medium, SUM149 cells and MCF10A AR cells in SFIH medium, and MCF10A + AR cells in SFIHA medium were added to the upper chamber of both control and rehydrated Matrigel invasion chambers. 5% fetal bovine serum was added to media in the bottom chamber as a chemoattractant. After 24 h, membranes were fixed and stained using the Hema 3 Staining System (Fisher). Membranes were allowed to dry and then were placed onto slides for visualization. Cells on control and Matrigel membranes were counted from three microscopic fields after 24 h. Percentage of invasion was calculated by dividing the mean number of cells on the invasion membranes by the mean number of cells on the control membranes for each cell line. Experiments were repeated three times.

Affymetrix Expression Array and Analysis—Total RNA was isolated from subconfluent cell culture plates using the RNeasy kit (Qiagen). The 28 and 18 S ribosomal RNA peak ratios were determined using microfluidics technology at the Wayne State University Applied Genomics Technology Center. The 28 S/18 S ratios of the RNA were determined to be higher than 1.3. 5–8 μg of RNA was then used for cDNA synthesis (Invitrogen). cRNA amplification was then performed using a kit from Enzo Diagnostics Inc. The cleaned cRNA was then hybridized to an Affymetrix human genome HGU133A array platform with 20,000 genes/chip. The expression array chip was scanned using an Agilent GeneArray scanner. The -fold change of fluorescence intensities was calculated relative to MCF10A. Analysis of array data was performed using Pathways-Express (39) and the Database for Annotation, Visualization, and Integrated Discovery (40).

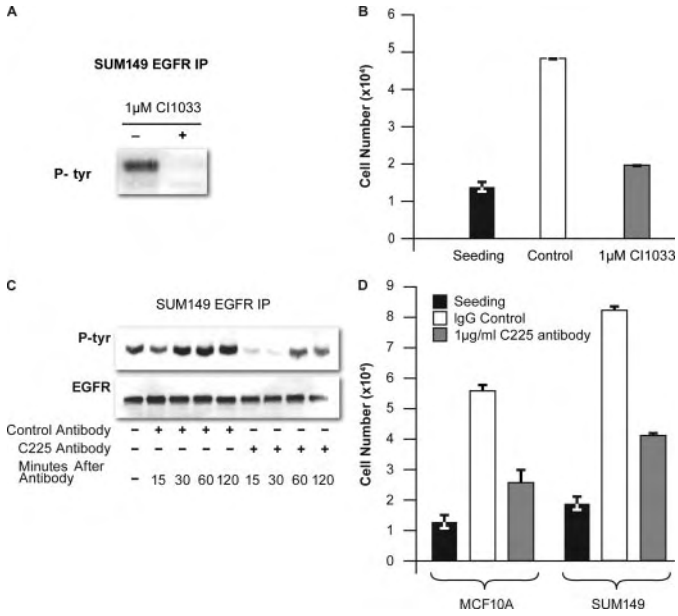

FIGURE 1. **SUM149 cells require ligand binding for EGFR activity and cell proliferation.** *A*, Western blot after EGFR immunoprecipitation (*IP*) showing EGFR tyrosine phosphorylation (*P-tyr*) after SUM149 cells were treated for 1 h with 1 μM CI1033. *B*, SUM149 cell counts after treatment with 1 μM CI1033 every day for 5 days. Cells were lysed, and the nuclei were counted using a Coulter Counter. *Error bars* represent the S.E. of three replicate experiments. *C*, Western blot after EGFR immunoprecipitation showing EGFR tyrosine phosphorylation in SUM149 cells after treatment with either 1 μg/ml isotype control IgG antibody or 1 μg/ml C225 antibody for the times indicated. *D*, MCF10A and SUM149 cell counts after treatment with 1 μg/ml C225 antibody every day for 5 days. Cells were lysed, and the nuclei were counted using a Coulter Counter. *Error bars* represent the S.E. of three replicate experiments.

RESULTS

SUM149 Cells Require Ligand Activation of EGFR for Cell Proliferation—Constitutive EGFR signaling in cancer cells contributes to aberrant cell proliferation and tumor progression. Our laboratory has shown previously that EGF-independent SUM149 breast cancer cells overexpress constitutively active EGFR protein but do not express any other active ErbB family members (41). Therefore, we utilized the pan-ErbB small molecule inhibitor CI1033 to determine whether SUM149 proliferation is dependent on EGFR activity. CI1033 prevents ATP binding in the kinase domain of the EGFR, thereby preventing activation of the receptor (42). EGFR tyrosine phosphorylation was profoundly reduced in SUM149 cells treated with 1 μM of CI1033 for 1 h (Fig. 1*A*), which is in agreement with published data (41). Moreover, treatment with 1 μM CI1033 every day for 5 days almost completely inhibited SUM149 cell proliferation (Fig. 1*B*). Therefore, SUM149 cells require EGFR activity for proliferation despite their EGF independence.

Growth factor independence is a hallmark of malignancy, and one mechanism for this phenotype is the development of autocrine loops in cancer cells. Therefore, it is possible that SUM149 cells synthesize their own ligand in an autocrine fashion, which is necessary to maintain EGFR phosphorylation. To determine whether the constitutive EGFR activity in SUM149 cells can be attributed to ligand activation of the receptor, SUM149 cells were incubated for 15-min, 30-min, 1-h, or 2-h time periods with the clone 225 (C225) mouse monoclonal antibody to EGFR. The C225 antibody neutralizes EGFR activ-

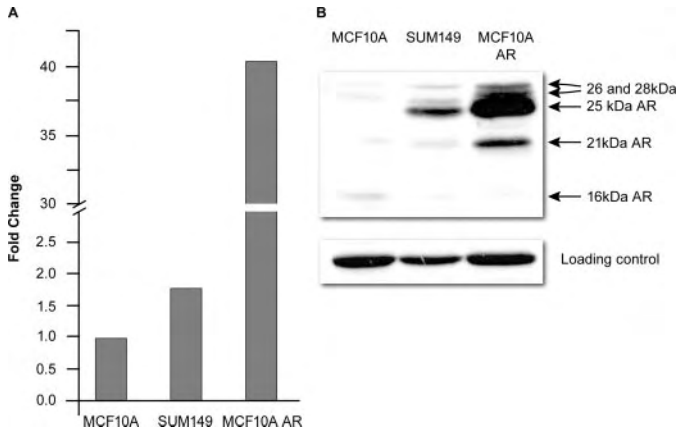

FIGURE 2. **AR is expressed at the mRNA and membrane protein levels in SUM149 cells.** *A*, AR mRNA was measured by quantitative RT-PCR in SUM149 cells and MCF10A AR cells. The $\Delta\Delta CT$ values were calculated as described under "Experimental Procedures" and graphed showing -fold change relative to MCF10A cells. Each experiment was performed in triplicate. *B*, Western blot showing AR protein after whole cell lysis of MCF10A, SUM149, and MCF10A AR cells. The anti-AR antibody AF262 was used to probe for AR.

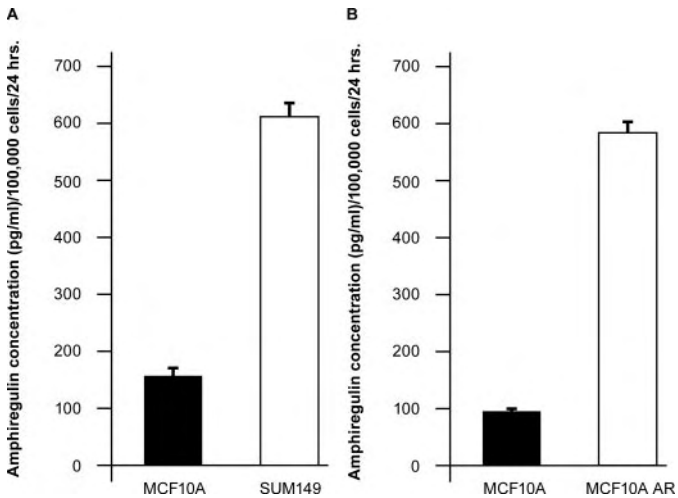

FIGURE 3. **Secreted AR is increased in the conditioned media from SUM149 cells (*A*) and MCF10A AR cells (*B*) relative to MCF10A cells.** MCF10A, SUM149, and MCF10A AR cells were cultured in 6-well plates with fresh medium, and the medium was harvested after 24 h. The amount of secreted AR in the conditioned medium was measured by an ELISA. Cells were counted, and the AR concentration was normalized to cell number. *Error bars* represent the S.E. of three replicate experiments.

ity by competing for the ligand binding domain of EGFR (43). We observed that treatment with C225 antibody resulted in complete attenuation of EGFR tyrosine phosphorylation at 15 and 30 min. At 1 and 2 h after antibody, EGFR tyrosine phosphorylation had increased but not to the levels of control (Fig. 1*C*). These data are consistent with our hypothesis that the EGFR in SUM149 cells exhibits a dependence on an autocrine ligand for activity.

To confirm that ligand activation of EGFR is required for SUM149 cell proliferation, a growth assay was performed in which the C225 antibody was added daily for 5 days and the cells were counted. The immortalized human mammary epithelial cell line MCF10A, which requires EGF for proliferation, was used as a positive control. The C225 antibody considerably reduced MCF10A cell proliferation, as expected. In addition, we found a significant reduction in SUM149 cell proliferation by growing cells in the presence of the antibody (Fig. 1*D*). The ability of C225 to decrease EGFR activity and cell proliferation suggests that endogenous EGFR ligands are regulating mitogenesis in SUM149 cells.

AR Is Overexpressed at the Message and Protein Levels in SUM149 Cells—Previous expression cloning experiments performed in our laboratory identified candidate genes expressed by breast cancer cells that mediate growth factor autonomy. Using this expression cloning strategy with a cDNA library derived from SUM149 cells, we found that overexpression of the EGF family member AR could transduce the EGF-independent phenotype to MCF10A cells (36). The AR-overexpressing MCF10A cells derived in these experiments (MCF10A AR cells) are EGF-independent for growth and have ~40-fold more AR mRNA than control MCF10A cells (Fig. 2*A*).

AR was the only EGFR ligand isolated from the SUM149 cDNA library that enabled MCF10A cells to grow without EGF (36). Since our data show that SUM149 cells are dependent on ligand activation of EGFR for proliferation, we hypothesized that SUM149 cells overexpress AR, which enables their EGF independence. To begin to test this hypothesis, we measured AR mRNA expression in SUM149 cells relative to MCF10A cells using quantitative RT-PCR analysis and discovered that SUM149 cells overexpress AR mRNA at levels ~2-fold higher than MCF10A cells (Fig. 2*A*).

Although the level of AR message is not dramatically higher in SUM149 cells relative to MCF10A cells, this difference in message contributes to a more notable difference in AR protein between the two cell lines (Fig. 2*B*). Following whole cell lysis and Western blot analysis, we observed a 16- and 21-kDa AR form as well as a 26- and 28-kDa doublet in all three cell lines, which is in agreement with previously described AR membrane precursors in Madin-Darby canine kidney cells after wild type AR overexpression (11, 44). However, SUM149 cells and MCF10A AR cells overexpress the 21-, 26-, and 28-kDa forms of AR protein relative to MCF10A cells. It is notable that a 25-kDa form, which has been suggested to be a less glycosylated version of the 26-kDa AR, was the predominant AR form in both SUM149 and MCF10A AR cells (11). In stark contrast, the 25-kDa form was not detectable in MCF10A cells, suggesting that this form in particular may play a significant role in EGF independence.

An ELISA was performed to quantitate secreted AR protein present in the conditioned media of SUM149 and MCF10A AR cells. Our results show that SUM149 cells secrete ~600 pg/ml AR into the medium/100,000 cells over a 24-h period, whereas MCF10A cells secrete ~150 pg/ml AR/100,000 cells over 24 h (Fig. 3*A*). MCF10A AR cells secrete about 6-fold more AR than MCF10A cells alone (Fig. 3*B*). Taken together, these data show that AR is overexpressed at the message level as well as the membrane and secreted protein levels in EGF-independent SUM149 breast cancer cells and MCF10A AR cells and therefore may be permitting SUM149 EGF independence via an autocrine loop.

Autocrine AR Is Required for EGFR Activity and SUM149 Cell Proliferation—AR was originally described as a bifunctional growth modulator, since it has both mitogenic and growth-inhibitory properties. Whether AR acts as a mitogen or a

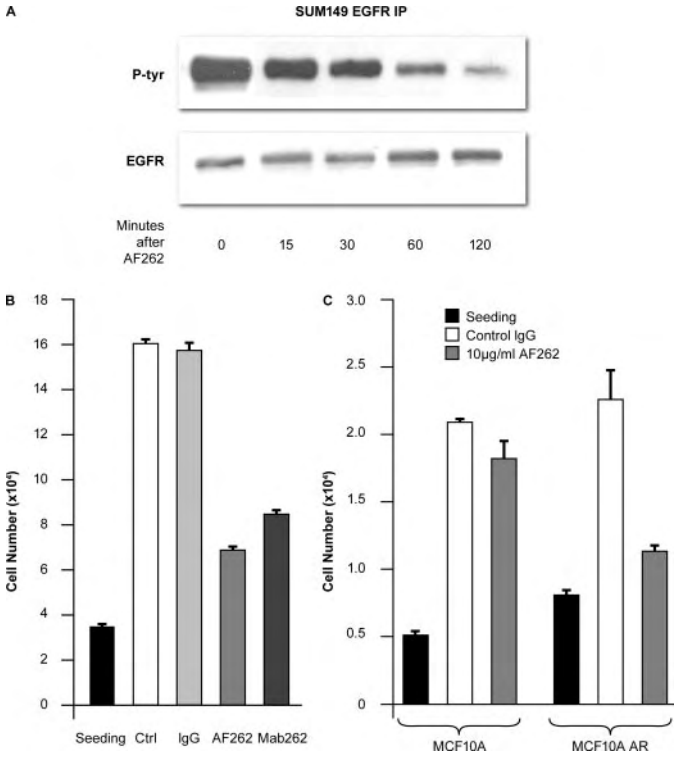

FIGURE 4. **AR-neutralizing antibody inhibits cell proliferation in SUM149 cells and MCF10A AR cells.** *A*, Western blot after EGFR immunoprecipitation (*IP*) showing EGFR tyrosine phosphorylation (*P-tyr*) in SUM149 cells after treatment with 1 μg/ml AR-neutralizing antibody AF262 for the times indicated. *B*, SUM149 cell counts after treatment with either 1 μg/ml isotype control IgG antibody, 1 μg/ml AR antibody AF262, or 1 μg/ml AR antibody MAB262 every day for 7 days. *Error bars* represent the S.E. of three replicate experiments. *C*, MCF10A and MCF10A AR cell counts after treatment with either 1 μg/ml isotype control IgG antibody or 1 μg/ml AR antibody AF262 every day for 7 days. *Error bars* represent the S.E. of three replicate experiments.

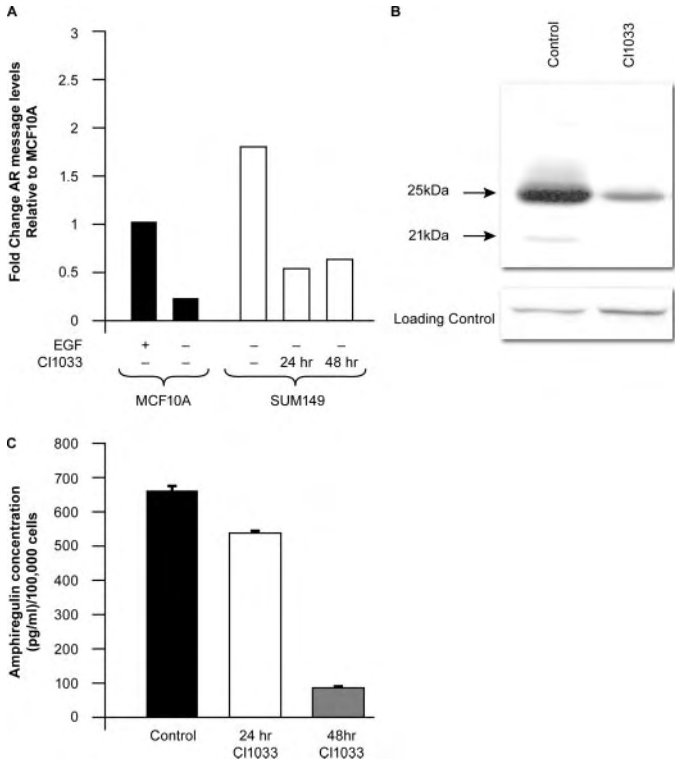

FIGURE 5. **AR expression is regulated by EGFR activity in SUM149 cells.** *A*, MCF10A and SUM149 AR mRNA was measured by quantitative RT-PCR after withdrawal of EGF for 24 h in the MCF10A cells or treatment of SUM149 cells with 1 μM CI1033 for 24 or 48 h. The $\Delta\Delta CT$ values were calculated as described under "Experimental Procedures" and graphed showing -fold change relative to MCF10A cells. *B*, whole cell lysis Western blot showing AR protein in SUM149 cells after treatment for 24 h with 1 μM CI1033. *C*, SUM149 cells were cultured in 6-well plates with fresh medium, and 1 μm CI1033 was added for 24 or 48 h. The medium was harvested after 24 h for control and 24-h treatments, and medium was harvested after 48 h for the 48-h treatment. The concentration of secreted AR in the conditioned medium was measured by an ELISA. *Error bars* represent the S.E. of three replicate experiments.

growth inhibitor is dependent upon several factors, including the concentration of AR and the nature of the target cells (19, 39). Therefore, it was critical to confirm that AR is the autocrine ligand required to activate EGFR in SUM149 cells. To address this question, two neutralizing antibodies were used to sequester AR. Western analysis following an EGFR immunoprecipitation demonstrated that 15 min after the addition of the AF262 AR-neutralizing antibody (R & D Systems), EGFR tyrosine phosphorylation was reduced, and phosphorylation continued to decrease in a time-dependent fashion (Fig. 4*A*). SUM149 cell proliferation was also significantly reduced following the addition of both neutralizing antibodies daily for 7 days (Fig. 4*B*). Furthermore, MCF10A AR cells showed almost a 3-fold reduction in proliferation relative to control cells when the AR neutralizing antibody was added (Fig. 4*C*). Thus, SUM149 cells and MCF10A AR cells are dependent on AR for their EGF-independent proliferation. MCF10A cells, which express AR mRNA and detectable AR protein but still require exogenous EGF for growth, showed slightly reduced cell proliferation with AR-neutralizing antibody (Fig. 4*C*). This result suggests that secreted AR contributes in a small way to the growth of MCF10A cells that takes place in EGF-containing medium.

To determine whether SUM149 EGFR activity contributes to the synthesis of AR, which would perpetuate the maintenance of an AR autocrine loop, CI1033 was added to SUM149 cells for 24 or 48 h to inhibit EGFR activity, and the effect on AR message and protein was determined. After 24 and 48 h following treatment with 1 μM CI1033, AR mRNA levels in SUM149 cells were reduced 3.7- and 3.8-fold, respectively (Fig. 5*A*). In addition, CI1033 significantly reduced AR membrane precursor protein and reduced secreted AR protein by over 6-fold in the SUM149 cells (Fig. 5, *B* and *C*). Thus, the EGFR system in SUM149 cells is autoinductive in that EGFR activation by AR is required for AR gene transcription. As expected, based on published results, the removal of EGF resulted in an approximately 3.5-fold decrease in AR mRNA levels, showing that AR is also regulated by EGFR in MCF10A cells (Fig. 5*A*) (26).

These data clearly demonstrate that AR is an autocrine growth stimulator for SUM149 breast cancer cells. Taken together with the observation that EGFR activity is required for synthesis and secretion of AR, these findings confirm that SUM149 cells have a functional AR autocrine loop that enables their EGF independence. Similar to SUM149 cells, MCF10A cells synthesize their own AR, which is dependent on EGFR activity. However, contrary to SUM149 cells, MCF10A cells are not able to maintain a self-sustaining AR autocrine loop in the absence of exogenous EGF. Rather, MCF10A cells depend on the cooperation of both EGF and AR for activation of EGFR and cell proliferation.

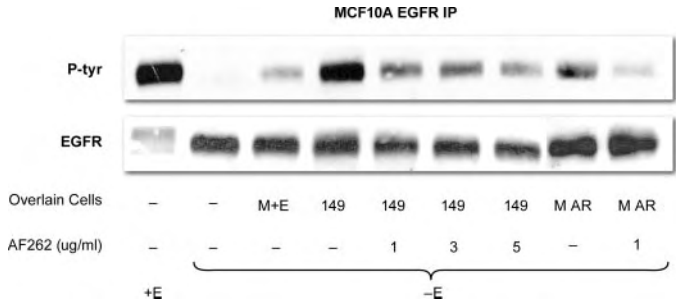

FIGURE 6. **Membrane precursor AR can activate EGFR in a juxtacrine fashion.** SUM149, MCF10A, or MCF10A AR cells were removed from confluent plates, acid-washed three times, fixed with formalin, and resuspended in SFIH medium. The cells were incubated with or without AR-neutralizing antibody AF262 for 5 min at concentrations of 1, 3, or 5 μg/ml as indicated. Fixed cells were then overlaid on top of EGF-starved MCF10A cells for 5 min. Western blot shows EGFR tyrosine phosphorylation (*P-tyr*) after an MCF10A EGFR immunoprecipitation.

AR Membrane Precursor Protein Can Activate EGFR via Juxtacrine Interactions—Pro-AR is a membrane-anchored precursor that is either cleaved to produce a soluble form that can activate EGFR or may possibly signal via juxtacrine interactions with EGFR (15, 45). It is still not clear whether the AR precursor is biologically active, but there is some evidence to suggest that AR can signal in a juxtacrine fashion (15). SUM149 breast cancer cells express significant amounts of membrane-bound AR, which may contribute to their ability to maintain an AR autocrine loop if the precursor form is biologically active. Therefore, we performed studies to investigate the potential mechanism of EGFR activation by membrane-bound AR.

To determine whether uncleaved AR is able to activate EGFR in a juxtacrine fashion, we performed a juxtacrine assay modified from the assay originally performed by Takemura *et al.* (46), which examined the signaling ability of membrane precursor HB-EGF. MCF10A cells, which express relatively high levels of EGF receptors, were grown without EGF for 24 h to decrease EGFR tyrosine phosphorylation. SUM149 and MCF10A AR cells were detached from confluent plates using EDTA and then acid-washed three times to remove cleaved cell-associated AR, leaving only membrane precursor AR on the cell surface. Following the acid washes, SUM149 and MCF10A AR cells were formalin-fixed and overlaid on top of the EGF-starved MCF10A cells for 5 min. Subsequent immunoprecipitation of EGFR followed by SDS-PAGE separation and immunoblotting with a phosphotyrosine antibody showed dramatically increased tyrosine phosphorylation of EGFR following exposure to fixed SUM149 cells and MCF10A AR cells but not with MCF10A cells that had been grown in their normal EGF-containing medium (Fig. 6). When AR-neutralizing antibody was added to the overlaid SUM149 cells and MCF10A AR cells, EGFR tyrosine phosphorylation was significantly reduced, suggesting that membrane precursor AR in both SUM149 cells and MCF10A AR cells activated EGFR on MCF10A cells via juxtacrine interactions (Fig. 6).

AR Cleavage Is Required for Autocrine Activation of EGFR in SUM149 Cells—It is noteworthy that our juxtacrine experiments demonstrate that membrane precursor AR can activate EGFR when the AR precursor is on cells laid on top of cells expressing EGFR. However, it is important to determine whether membrane precursor AR or cleaved AR is the most important for the maintenance of an autocrine loop when the cells are growing adjacent to each other in monolayer. In an effort to determine the requirement of AR cleavage for EGFR activity in SUM149 cells, SUM149 cells were treated with the broad spectrum metalloprotease inhibitor GM6001, which has been shown to block AR cleavage in keratinocytes (47).

To confirm that GM6001 inhibits AR secretion in SUM149 cells, an ELISA was performed. SUM149 cells were subjected to one, two, or three acid washes to remove cleaved AR attached to the heparan sulfate proteoglycan surface of the cell, which could be released into the medium and increase the total background AR concentration. These acid washes were then followed with Me_2SO control treatment or 20 μM GM6001 for 5, 15, or 30 min. As observed with the Me_2SO controls, each consecutive acid wash removed more residual cleaved AR from the medium. After three acid washes and GM6001 treatment, secreted AR in SUM149 conditioned medium dropped to a concentration below 5 pg/ml/100,000 cells (Fig. 7*A*). As a result, these data show that AR secretion is essentially completely blocked by GM6001 in SUM149 cells.

In an effort to determine how blocking AR secretion affects EGFR activity, we acid-washed MCF10A, MCF10A AR, and SUM149 cells three times to remove cleaved AR from the cell surface and then exposed cells to 20 μM GM6001 for varying times. GM6001 had no effect on exogenous EGF-induced tyrosine phosphorylation of EGFR, since EGFR phosphorylation was not reduced by GM6001 treatment of MCF10A cells cultured in the presence of EGF (Fig. 7*B*). However, inhibition of AR secretion with GM6001 in SUM149 and MCF10A AR cells resulted in a significant decrease in EGFR tyrosine phosphorylation with very little residual phosphorylation remaining at 60 min (Fig. 7*C*).

Given that AR cleavage was effectively blocked by GM6001 after only 5 min, it was possible that the residual EGFR activity we observed from 15 to 60 min after GM6001 treatment in SUM149 and MCF10A AR cells was induced by the noncleaved AR precursor on the cell surface. Alternatively, noncleaved AR may have no ability to activate EGFR in a truly autocrine fashion, in which case the residual EGFR tyrosine phosphorylation observed would represent activated receptor that had not been completely degraded or dephosphorylated. To distinguish between these possibilities, MCF10A cells were cultured in AR-supplemented serum-free medium. We then examined how quickly EGFR activity decreases after removal of exogenous AR from the medium. MCF10A cells were grown in 10 ng/ml AR for one passage, and AR was washed out for 5, 15, 30, or 60 min, followed by EGFR immunoprecipitation and Western analysis (Fig. 7*D*). We found that EGFR tyrosine phosphorylation, after removal of soluble AR, decreased in a fashion similar to EGFR tyrosine phosphorylation in SUM149 and MCF10A AR cells after treatment with GM6001. Thus, the residual EGFR tyrosine phosphorylation we observed after GM6001 treatment appears to be activated receptor that had not been completely degraded or dephosphorylated. Altogether, our data demonstrate that whereas membrane precursor AR can activate EGFR via juxtacrine interactions, cleavage of AR plays a critical role in autocrine activation of EGFR.

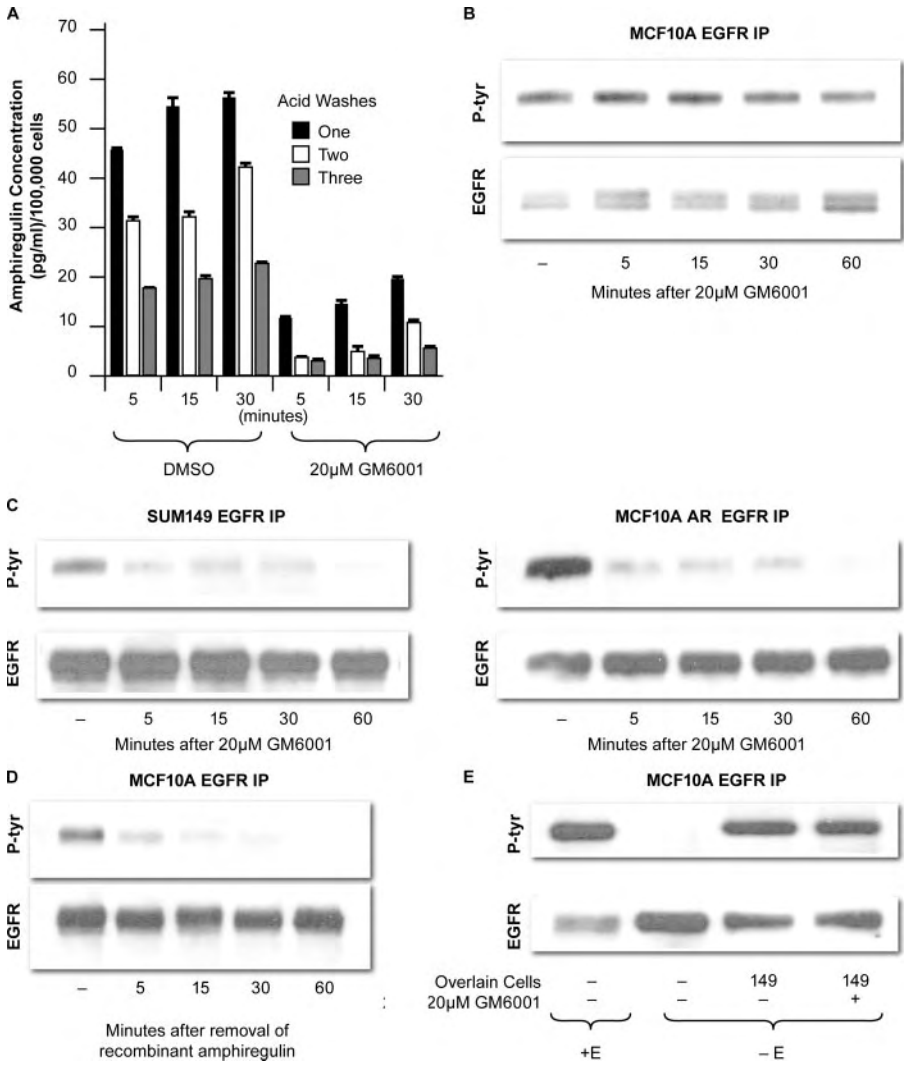

FIGURE 7. **AR cleavage is required for an autocrine loop in SUM149 cells and MCF10A AR cells.** *A*, SUM149 cells were cultured in 6-well plates with fresh medium. Cells were acid-washed once, twice, or three times and treated with either Me_2SO (*DMSO*) or 20 μM GM6001 for 5, 15, 30, or 60 min. Conditioned medium was measured for AR by an ELISA. Cells were counted for normalization using a Coulter Counter. *Error bars* represent the S.E. of three replicate experiments. *B*, Western blot after EGFR immunoprecipitation showing EGFR tyrosine phosphorylation (*P-tyr*) in MCF10A cells that were acid-washed three times and treated with 20 μM GM6001 for 5, 15, 30, or 60 min. *C*, Western blot after EGFR immunoprecipitation showing EGFR tyrosine phosphorylation in SUM149 and MCF10A AR cells that were acid-washed three times and treated with 20 μM GM6001 for 5, 15, 30, or 60 min. *D*, Western blot after EGFR immunoprecipitation showing EGFR tyrosine phosphorylation in MCF10A cells that were grown in SFIHA medium for one passage. AR was washed away, and the cells were cultured in SFIH medium for 5, 15, 30, or 60 min. *E*, juxtacrine assay after SUM149 cells were treated with 20 μM GM6001 for 15 min.

The requirement for cleavage for autocrine activation of EGFR seemed incongruous with the observation that membrane precursor AR can activate EGFR in our juxtacrine assay. Together, these observations suggest that AR cleavage is required for autocrine activation of the EGFR but not for juxtacrine activation of EGFR. To examine this further and confirm that noncleaved, membrane-associated AR is able to activate EGFR, we repeated the juxtacrine experiment after blocking AR secretion with three acid washes followed by GM6001 for 15 min in the SUM149 cells. The data in Fig. 7*E* shows that inhibition of AR secretion using the metalloprotease inhibitor GM6001 did not effect activation of MCF10A EGFR by overlaid SUM149 cells. Thus, we conclude that the increased EGFR tyrosine phosphorylation observed in MCF10A cells overlaid with SUM149 or MCF10A AR cells is due to AR membrane precursor that interacts with and activates the EGFR.

Overexpression of AR Increases Directional Motility and Invasion of MCF10A Cells—One of the hallmark characteristics of cancer cells is enhanced motility. To determine how an AR autocrine loop might contribute to the aggressive breast cancer phenotype, we investigated the effect of EGFR activation by AR on cell motility. We utilized a fluorescence cell motility assay in which the cells phagocytose blue fluorescent beads in their path, leaving black tracks behind as they move (Cellomics). We found that SUM149 cells, which overexpress AR, displayed an increase in motility compared with MCF10A cells (Fig. 8*A*). In addition, both exogenous AR (MCF10A + AR) and overexpression of AR (MCF10A AR) significantly stimulated directional motility compared with MCF10A cells grown in EGF-containing medium, suggesting that an AR autocrine loop specifically contributes to the activation of genes involved in cell motility.

Penetration of the extracellular matrix and the basement membrane is a key step in tumor dissemination and invasion. Therefore, we investigated the effect of AR on cell invasion in a modified Boyden chamber assay in which cells migrate through Matrigel and a polycarbonate filter (BD Biosciences). We evaluated the invasive capacity of SUM149 cells, MCF10A AR cells, and MCF10A cells grown with either exogenous EGF or exogenous AR by determining the number of cells that penetrated the Matrigel. We found that MCF10A cells grown with EGF lack the ability to invade, whereas MCF10A cells grown with AR were ~25% invasive. In addition, MCF10A AR cells were close to 35% invasive, suggesting that AR activation of EGFR, but not EGF activation of EGFR, increases cell invasion. SUM149 cells were ~50% invasive in this assay (Fig. 8*B*). Therefore, these data strongly indicate that an AR autocrine loop specifically contributes to the aggressive growth potential of SUM149 breast cancer cells.

SUM149 Cells and MCF10A AR Cells Express Common Genes Related to Cell Motility and Invasion—In an effort to determine which genes might be contributing to the increased cell invasion and motility of MCF10A cells growing with AR-

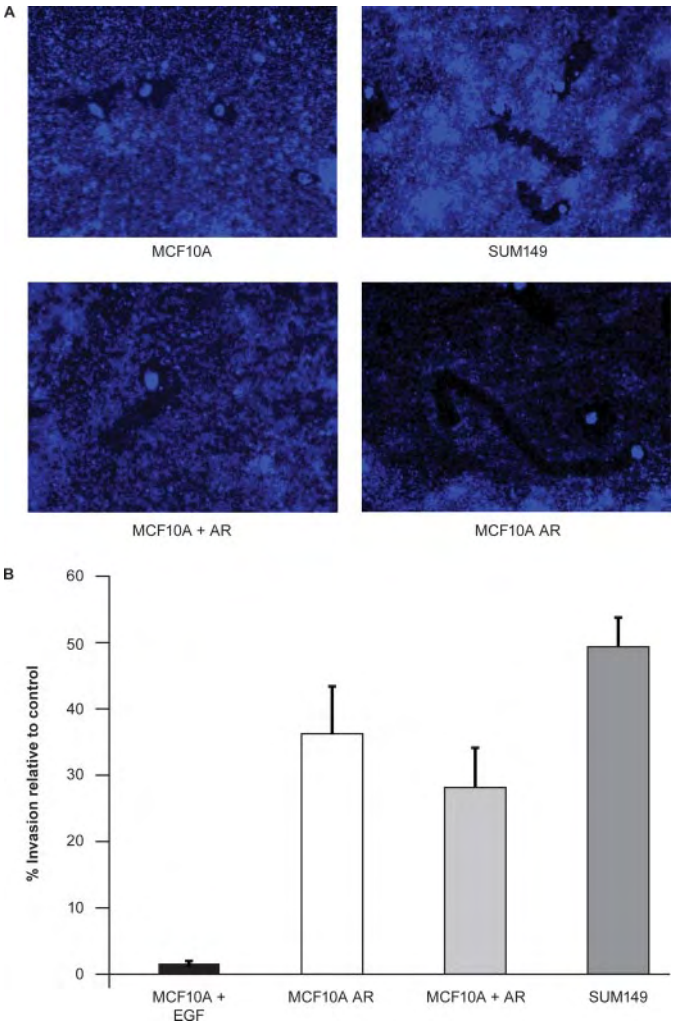

FIGURE 8. **AR activation of EGFR increases cell motility and invasion.** *A*, cell motility assay images showing *black tracks* made by MCF10A cells grown in EGF (MCF10A), MCF10A cells grown in AR (MCF10A + AR), SUM149 cells, and MCF10A AR cells. Pictures were taken at ×20 magnification after 24 h. *B*, percentage of invasion of MCF10A cells grown in EGF (MCF10A), MCF10A cells grown in AR (MCF10A + AR), SUM149 cells, and MCF10A AR cells was calculated by dividing the average number of cells on the membrane of a Matrigel chamber after 24 h by the average number of cells on the membrane of a control chamber after 24 h. *Error bars* represent the S.E. of three replicate experiments.

stimulated EGFR, we performed an expression array analysis using an Affymetrix human array platform comparing MCF10A cells *versus* SUM149 cells or MCF10A AR cells. Analysis of the results indicated that 97 genes are increased in their expression in both SUM149 and MCF10A AR cells relative to MCF10A cells (data not shown). Further analysis of the biological pathways using the data mining tool Pathway-Express demonstrated that most of these genes fell into the major biological processes of Toll-like receptor signaling pathway, apoptosis, mitogen-activated protein kinase signaling pathway, focal adhesion, and cytokine-cytokine receptor interaction (39). Annotation analysis using the Database for Annotation, Visualization, and Integrated Discovery showed that 14 genes of the 97 up-regulated genes were involved in processes such as cell adhesion, proteolysis, and chemotaxis that are related to cell motility and invasion (Table 1) (40). Our data suggest that these genes may play particularly important roles in the breast cancer phenotype when cancer cells are dependent on a functional AR/EGFR autocrine loop.

DISCUSSION

AR is involved in an autocrine loop in several types of cancers, such as colon cancer, pancreatic cancer, and hepatocellular carcinoma, and it is presumed that an AR/EGFR autocrine loop also contributes to breast cancer progression (27–29). There is significant evidence to support an autocrine loop in breast cancer, since levels of AR protein expression are generally higher in invasive breast carcinomas than in ductal carcinoma *in situ* (DCIS) or in normal mammary epithelium (30, 48, 49). Also, AR antisense strongly reduced tumorigenicity of transformed breast epithelial cells *in vivo* (50). However, currently, there is no direct evidence of an amphiregulin autocrine loop in breast cancer cells. The present study demonstrates directly that there is a self-sustaining AR autocrine loop functioning in SUM149 human breast cancer cells, which contributes to the aggressive breast cancer phenotype.

SUM149 breast cancer cells are a widely used model of aggressive breast cancer, and they are representative of a subset of breast cancers that have EGFR overexpression and estrogen receptor negativity (36, 41, 51–53). AR is constitutively expressed and released into the conditioned medium of SUM149 cells on which it exerts promitogenic effects through activation of EGFR. Indeed, inhibition of AR shedding or neutralization of AR resulted in reduced EGFR activation and cell proliferation. Interestingly, we also observed that AR was able to stimulate its own gene expression in SUM149 cells through the activation of EGFR; thus, EGFR activity is absolutely critical to AR synthesis. A similar response has been observed in colon cancer cells and hepatocellular carcinoma cells, where AR synthesis is induced by EGFR activity (27, 28). Altogether, it is apparent that SUM149 cells have a self-perpetuating AR autocrine loop.

In stark contrast, the normal human mammary epithelial cell line MCF10A does not have a self-sustaining AR autocrine loop. Although these cells synthesize AR mRNA at similar levels to SUM149 cells, they are still dependent on EGF for EGFR activity. If EGF is removed from the medium, AR message decreases, and the AR autocrine loop breaks down. We observed that the AR-neutralizing antibody partially inhibited MCF10A cell proliferation even as the MCF10A cells were growing with exogenous EGF in the medium, suggesting that AR and EGF both participate in activation of EGFR. Therefore, these two ligands function as cooperating growth factors in these cells. Ligand cooperation has been previously noted in other immortalized human mammary epithelial cells that were shown to synthesize and respond to TGF-α but still required EGF for EGFR activity and cell growth (54, 55).

SUM149 cells do not require exogenous EGF for growth. In addition, the only other EGF family ligand they synthesize is epiregulin at the mRNA level (data not shown). It has been shown previously that AR and epiregulin are often co-expressed. Epiregulin and AR are located within 100 kb of each other on chromosome 4q13-21, and therefore these genes may be co-regulated (21, 56). However, we did not observe any response by SUM149 cells to epiregulin-neutralizing antibody

TABLE 1
Differentially regulated genes involved in cell motility and invasion

Gene accession number	Gene name	Change	Annotation
		-fold	
NM_005823	MSLN (mesothelin)	55.72	Cell adhesion
BC012501	NID (nidogen)	19.70	Cell matrix adhesion; extracellular matrix structural constituent; calcium ion binding; cell adhesion molecule activity
M15329	IL1A (Interleukin 1α)	18.38	Negative regulation of cell proliferation; anti-apoptosis; chemotaxis; immune response; regulation of cell cycle; cell-cell signaling; inflammatory response; interleukin-1 receptor binding; signal transducer activity
AI922855	CPE (carboxypeptidase 3)	11.31	Metabolism; protein modification; proteolysis and peptidolysis; neuropeptide signaling pathway; metallopeptidase activity
NM_006307	SRPX (sushi-repeat-containing protein X-linked)	5.66	Cell adhesion
AW002864	AAD2 (adducin 2β)	5.66	Actin binding; calmodulin binding
U07820	CNTN1 (contactin 1)	5.28	Cell adhesion
NM_000576	IL1B (interleukin 1β)	4.92	Negative regulation of cell proliferation; apoptosis; immune response; signal transduction; regulation of cell cycle; cell-cell signaling; inflammatory response; antimicrobial humoral response; interleukin-1 receptor binding; signal transducer activity
NM_024022	TMPRSS3 (transmembrane protease, serine 3)	4.92	Hearing; proteolysis and peptidolysis; trypsin activity; scavenger receptor activity; chymotrypsin activity; hydrolase activity
BC002690	KRT14 (keratin 14)	4.29	Epidermal differentiation; cell shape and cell size control; structural constituent of cytoskeleton
AI653981	L1CAM (cell adhesion molecule)	2.64	Neurogenesis; cell adhesion molecule activity
U61276	JAG1 (Jagged 1)	2.14	Cell communication; Notch binding; calcium ion binding; growth factor activity; structural molecule activity
NM_006255	PRKCH (protein kinase C, eta)	2.14	Protein amino acid phosphorylation; signal transduction
NM_002423	MMP7 (matrix metalloprotease 7)	2.00	Collagen catabolism; zinc ion binding; calcium ion binding; matrilysin activity; hydrolase activity

(data not shown). Since AR-neutralizing antibody almost completely blocked proliferation of SUM149 cells, it can be concluded that AR is the only EGF family ligand that is required for their EGFR activation.

We have shown previously that MCF10A AR cells, like SUM149 cells, are EGF-independent for proliferation (36). However, the most compelling phenotypic alterations specifically induced by AR in MCF10A cells were an increase in cell motility and invasion. Increased motility and the ability to invade the basement membrane are characteristic of transformed cells (57). Although the mechanisms that allow this change in biology with AR signaling and not EGF signaling are still unclear, it is apparent that EGF and AR ligands can promote dramatically distinct patterns of gene expression. We discovered several genes involved in cell motility and invasion that were up-regulated when MCF10A cells were grown in AR *versus* EGF. In addition, many of these genes were also up-regulated in the SUM149 breast cancer cells.

The unique role of AR in cell motility and invasion has been suggested in previous studies. For example, it has been shown that AR increases the invasion of MCF-7 and MDA-MB231 breast cancer cells in Matrigel (31). AR also apparently plays a role in actin rearrangement through the redistribution of E-cadherin, which may have an affect on cell motility (58, 59). In light of these observations, we propose that AR exerts a distinct, nonredundant phenotype in HME cells that cannot be compensated for entirely by other EGF family member ligands. This theory is supported by previous data demonstrating that AR and other EGF family ligands have different affects on ductal outgrowth in the mammary gland (25). The reasons for this predominant role by AR in cell motility and invasion are not clear at present and deserve further investigation.

Consistent with previous studies demonstrating that AR precursor can activate EGFR in a juxtacrine fashion, we showed that the membrane precursor AR from SUM149 and MCF10A AR cells can activate EGFR when overlaid on top of MCF10A cells, and this activation can be reduced substantially using an AR-neutralizing antibody (15). It appears contradictory that we found that membrane precursor AR can activate EGFR via juxtacrine interactions but cleaved AR appears to be required for maintenance of an autocrine loop in SUM149 cells and MCF10A AR cells growing in culture. These data suggest that localization of EGFR may be a contributing factor in the ability of AR and EGFR to signal in a juxtacrine fashion. Temporal and spatial control of EGFR signaling has been observed previously as a way in which the biological response of the cell is controlled (60). In cell culture where cells are in a monolayer, it is possible that cleavage is required due to a spatial restriction that prevents membrane precursor AR from coming into contact with the EGFR. Therefore, juxtacrine interactions may play more of a role *in vivo* in a three-dimensional tumor where cells are nonpolarized and EGFR trafficking is dysregulated.

In summary, we demonstrate that AR is the predominant EGF-like ligand produced by SUM149 breast cancer cells, and it regulates their growth in an autocrine manner. We have implicated a self-sustaining AR loop in breast cancer progression based on its ability to promote cell proliferation, motility, and invasion, which are all essential characteristics of aggressive cancer cells. Finally, juxtacrine activation of EGFR by AR membrane precursor protein may contribute to the maintenance of growth factor independence of a breast tumor, and therefore blocking AR cleavage with metalloprotease inhibitors could be ineffective at reducing proliferation of breast cancer cells in patients. Since EGFR inhibitors are only modestly effective in the clinic, targeting AR expression and activity may be more effective in overcoming EGFR inhibitor resistance of certain types of breast cancer (61, 62).

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